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Postlabeling Methods for Carcinogen–DNA Adduct Analysis

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Radioactive carcinogens have provided most of our present knowledge about the chemistry of interactions between carcinogens and biological systems. The requirement of radioactive carcinogens has restricted carcinogen–DNA binding studies to chemicals that are readily available in isotopically labeled form, i.e., a minute fraction of all potentially mutagenic or carcinogenic chemicals. To extend the scope of carcinogen–DNA binding studies, an alternative method, which does not require radioactive test chemicals, has been developed. In this approach, radioactivity (^{32}P) is being incorporated into DNA constituents by polynucleotide kinase-catalyzed [^{32}P]phosphate transfer from [γ-^{32}P]ATP after exposure of the DNA *in vitro* or *in vivo* to a nonradioactive, covalently binding chemical, and evidence for the alteration of DNA nucleotides is provided by the appearance of extra spots on autoradiograms of thin-layer chromatograms of digests of the chemically modified DNA. Quantitation of adduct levels is accomplished by scintillation counting. The sensitivity of the technique depends on the experimental conditions for ^{32}P-labeling and on the chemical structure of the adducts. Greater sensitivity may be achieved if adducts can be separated as a class from the normal nucleotides. This is the case for an estimated 80% of all carcinogens, giving rise to bulky and/or aromatic substituents in DNA. Under the present conditions, one such adduct in 10^9 to 10^{10} normal nucleotides can be detected. A total of approximately 80 compounds has been studied thus far. Binding to DNA of rodent tissues was readily detected by the ^{32}P-postlabeling assay for all known carcinogens among these compounds, and adducts were detected in DNA from human placenta of smokers.

Introduction

Any chemical capable of forming covalent bonds with DNA of somatic and reproductive mammalian cells *in vivo* is a potential mutagen, carcinogen, and teratogen. Since such genotoxic chemicals may be of natural or man-made origin, exposure to them cannot be completely eliminated, but human contact with them must be minimized. Methods enabling one to detect and quantify DNA binding potential of chemicals directly *in vivo* should be of great value in the detection of gene-altering chemicals in the environment, provided they can be applied to a large number of chemicals of diverse structure and their sensitivity is sufficient to detect low DNA-binding activities. Because covalent DNA binding of chemicals in experimental animals may range from one adduct in 10^8 normal nucleotides to one adduct in 10^9 to 10^{10} nucleotides, such methods should ideally be capable of detecting extremely low binding of the order of a single adduct per diploid mammalian genome of about 1.2×10^{10} DNA nucleotides.

As pointed out by Lutz (*1,2*), the covalent binding index (CBI), defined as μmole of chemical bound per mole of DNA nucleotide/millimole of chemical administered per kilogram body weight of animal, exhibits a good quantitative correlation with the hepatocarcinogenic potency of chemicals of diverse structure. Therefore, methods for the analysis of adducts in DNA should not only detect but also quantitate DNA binding. The subject of this paper is to review our recent efforts to develop such an ultrasensitive method involving ^{32}P-postlabeling of adducts (*3–6*) and to describe results obtained by applying the method to a number of genotoxic chemicals of diverse structure.

Materials and Methods

The sources of materials used in the ^{32}P-postlabeling procedure as well as safety precautions and chromatographic and autoradiographic procedures have been re-

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Table 1. Compounds tested for DNA binding *in vivo* by ^{32}P-postlabeling analysis.

Compound	Tissue[a]	DNA adducts	
		No.[b]	Levels[c]
Arylamines and derivatives			
2-Acetylaminofluorene	MS	6	+ +
	RL	11	+ + +
4-Acetylaminofluorene	RL	2	+
N-Hydroxy-2-acetylaminofluorene	RL	16	+ + +
N-Hydroxy-2-acetylaminophenanthrene	RL	10	+ + +
N-Hydroxy-4-acetylaminobiphenyl	RL	10	+ + +
N-Hydroxy-4-acetylamino-*trans*-stilbene	RL	9	+ + +
4-Aminobiphenyl	MS	1	+
Benzidine	MS	3	+
Azo compounds			
4-Dimethylaminoazobenzene	MS	2	+
Congo red	MS	2	+
Evan's blue	MS	1	+
Nitro compounds			
4-Nitroquinoline-1-oxide	MS	8	+ +
2,6-Dinitrotoluene	MS	3	+
Polycyclic aromatic hydrocarbons			
Benzo(a)pyrene	MS	5	+ + +
	RL	2	+ +
7,12-Dimethylbenz(a)anthracene	MS	8	+ + +
3-Methylcholanthrene	MS	13	+ + +
Benzo(e)pyrene	MS	5	+
Benz(a)anthracene	MS	2	+
Dibenz(a,c)anthracene	MS	6	+
Dibenz(a,h)anthracene	MS	3	+ +
Benzo(g,h,i)perylene	MS	2	+ +
Chrysene	MS	2	+ +
Anthracene	MS	ND[d]	−
Pyrene	MS/ML	ND[d]	−
Perylene	MS	ND[d]	−
Benzo(a)fluorene	MS	5	+
Benzo(b)fluorene	MS	5	+
Heterocyclic polycyclic compounds			
Dibenzo(c,g)carbazole	MS	7	+ + +
Dibenzo(a,i)carbazole	MS	6	+
Dibenz(a,j)acridine	MS	2	+ +
Alkenylbenzenes			
Safrole	ML	4	+ + +
Estragole	ML	4	+ + +
Methyleugenol	ML	4	+ + +
Myristicin	ML	3	+ + +
Dill apiol	ML	3	+ + +
Parsley apiol	ML	3	+ + +
Isosafrole	ML	2	+ +
Elemicin	ML	2	+ + +
Anethole	ML	2	+ +
Allylbenzene	ML	2	+ +
Methylating agents			
N,*N*-Dimethylnitrosamine	ML	5	+ + + +
1,2-Dimethylhydrazine	ML	5	+ + + +
N-Methyl-*N*-nitrosourea	ML	5	+ + + +
Streptozotocin	ML	5	+ + + +
Mycotoxins			
Aflatoxin B_1	RL	9[e]	+ + + +
Sterigmatocystin	RL	15[e]	+ + +

[a] MS, mouse skin; ML, mouse liver; RL, rat liver.
[b] These numbers reflect the total number of adducts detected, including those requiring very prolonged exposures for their detection.
[c] Total adduct levels; +, 1 adduct in $> 10^7$ nucleotides; + +, 1 adduct in 5×10^5–10^7 nucleotides; + + +, 1 adduct in 10^4–5×10^5 nucleotides; + + + +, 1 adduct in $< 10^4$ nucleotides.
[d] Not detected.
[e] These adducts are oligonucleotides containing covalently bound carcinogen (see text).

ported previously (*4,6*). [γ-^{32}P]ATP was synthesized in the laboratory as described (*4*). To remove background material from maps of aromatic carcinogen–DNA adducts, polyethyleneimine–cellulose (PEI–cellulose) thin layers were given a final development in 0.35 M $MgCl_2$ before autoradiography(*6*). Screen-intensified autoradiography was performed at −80°C. For *in vivo* modification of DNA, female BALB/c or CD-1 mice (25 g) and male Sprague-Dawley or Fischer rats (200 g) were maintained on standard laboratory diet and water *ad libitum*. A list of chemicals used for animal treatments is given in Table 1. For adduct detection in mouse skin DNA, the backs of mice were shaved with clippers 3 days prior to treatment, which was performed by topical application of four doses of 1.2 μmole each of test compounds in 200 μL of solvent. Compounds were dissolved in acetone, except for azo dyes, which were dissolved in acetone/water (7:3, v/v). The treatments were at 0, 24, 48, and 72 hr for arylamines and derivatives, azo compounds, and nitro compounds, and at 0, 6, 30, and 54 hr for polycyclic aromatic hydrocarbons. Control mice were given 200 μL of solvent alone. DNA was isolated 24 hr after the last treatment. For adduct detection in mouse liver DNA, animals were given a single IP dose (150 mg/kg) of methylating agent in 0.1 mL of 0.9% NaCl; control mice received 0.9% NaCl alone. DNA was extracted 3 hr after administration. Alkenylbenzenes were given to mice by IP injection of compound (400 mg/kg) in 0.2 mL tricaprylin, and liver DNA was isolated 24 hr after treatment; control mice received tricaprylin alone. Dibenzo(c,g)carbazole was given SC to female CD-1 mice at a dose of 12 mg/kg in tricaprylin. For adduct detection in rat liver DNA, animals were given a single IP dose of test chemical (40 mg/kg) in 0.3 mL of dimethyl sulfoxide; control rats received vehicle alone. DNA was isolated 4 hr after administration. Treatment with mycotoxins is detailed below. ^{32}P-postlabeling analysis of DNA adducts was performed as described previously (*4,6*) using 1 to 2 μg of DNA for enzymatic digestion and 0.15 to 0.3 μg of DNA nucleotides for ^{32}P-labeling. For adduct intensification, the labeling reaction mixture contained 400 μM DNA-P and 1.7 μM [γ-^{32}P]ATP (9120 Ci/mmole), and apyrase treatment (*4*) was omitted. For estimation of adduct levels, spots were excised from replicate maps and counted by Cerenkov assay. Appropriate blank areas of the chromatogram were also assayed and their count rates subtracted from the sample count rates. In the case of aromatic adducts, the amounts of ^{32}P-labeled digest applied to the thin-layer chromatograms were 150 to 460 μCi for counting of adducts and 0.25 μCi for assaying normal nucleotides. Calculations were done according to the equations shown in Figure 1. Under the conditions of excess ATP (*4,6*), the relative adduct labeling (RAL) accurately reflects the adduct levels in the samples analyzed, a RAL value of 10^{-7} corresponding to a DNA modification level of about 0.3 pmole of adduct/mg of DNA.

Adduct quantitation by ^{32}P-postlabeling assay

$$\text{Relative adduct labeling (RAL)} = \frac{\text{cpm}_{\text{Adduct(s)}}}{\text{cpm}_{\text{Normal nucleotides}} + \text{cpm}_{\text{Adducts}}}$$

$RAL \times 10^7$ = No. of adducts in 10^7 total nucleotides

$1/RAL$ = No. of total nucleotides per adduct

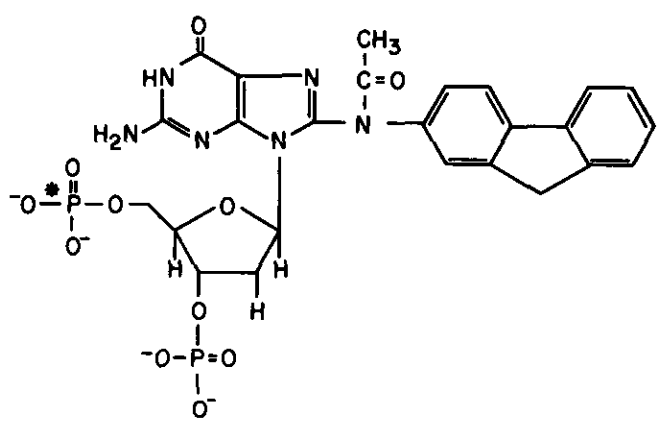
$0.3\ (RAL \times 10^7)$ = fmol adduct/μg DNA

FIGURE 1. Adduct quantitation involves determination of radioactivities of adducts and normal nucleotides by Cerenkov counting and calculation of RAL values as indicated. Adjustments are made for radioactive decay and dilutions of normal nucleotides for chromatography (*4,6*). If $\text{cpm}_{\text{Adducts}} << \text{cpm}_{\text{Normal nucleotides}}$, $\text{cpm}_{\text{Adducts}}$ is omitted from calculations of the denominator.

Results

For the ^{32}P-postlabeling scheme to serve as a test for the capacity of chemicals to bind to DNA, it should be applicable to most or all covalently binding chemicals; thus a major question to be answered in the initial phase of the development of the method was whether a greater number of adducts of diverse structure was amenable to ^{32}P-postlabeling by T4 polynucleotide kinase-catalyzed phosphorylation. We have thus far studied a total of ca. 80 chemicals comprising arylamines and derivatives, azo compounds, nitroaromatics, polycyclic aromatic hydrocarbons, heterocyclic polynuclear compounds, alkenylbenzenes, mycotoxins, and methylating agents. In every case, ^{32}P-labeling of carcinogen–DNA derivatives could be readily detected, indicating that the ^{32}P-postlabeling method can be applied to a very large number of chemically diverse adducts. Many of the compounds studied are listed in Table 1.

The ^{32}P-postlabeling procedure entails four consecutive steps (Fig. 2): digestion of modified DNA to 3′-mononucleotides; incorporation of ^{32}P into the latter; removal of normal nucleotides; and TLC separation and autoradiography of ^{32}P-labeled adduct nucleotides. These compounds are [5′-^{32}P]deoxyribonucleoside 3′,5′-



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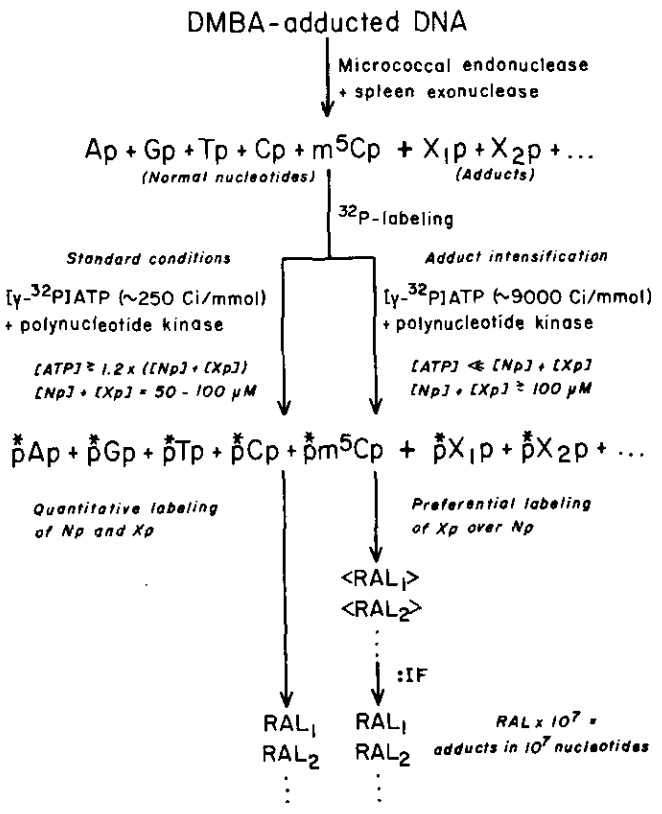

FIGURE 2. ^{32}P-postlabeling analysis of 7,12-dimethylbenz(a)anthracene (DMBA)–DNA or other aromatic adducts under standard or adduct intensification conditions. Adducted DNA is digested to normal (Ap, Gp, etc.) and adducted (X_1p, X_2p, etc.) deoxyribonucleoside 3′-monophosphates, which are converted to 5′-^{32}P-labeled deoxyribonucleoside 3′,5′-bisphosphates by polynucleotide kinase-catalyzed [^{32}P]phosphate transfer from [γ-^{32}P]ATP. All the substrate nucleotides are quantitatively ^{32}P-labeled in the presence of an excess of ATP (left-hand side), while under ATP-deficient conditions (right-hand side) the adducts are labeled to a greater extent than are the normal nucleotides. [Np] + [Xp] = [DNA-P] = Sum of concentrations of normal and adducted deoxyribonucleoside 3′-monophosphates. RAL and <RAL> values are calculated from the count rates of adducts and normal nucleotides (see Fig. 1). Individual <RAL> values are divided by experimentally determined intensification factors (IF's) to obtain RAL values.

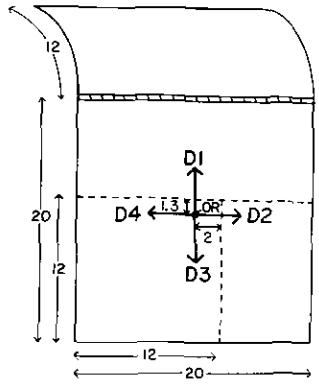

FIGURE 3. Diagram of four-directional (4-D) PEI–cellulose TLC for the separation of ^{32}P-labeled carcinogen–DNA adducts. A Whatman 1 wick is attached to the top of the PEI–cellulose thin layer, and ^{32}P-labeled DNA digest is applied at OR. Developments in direction 1 (D1) and D2 are in 1.1 M LiCl and 2.5 M ammonium formate (pH 3.5), respectively. These developments serve to remove ^{32}P-labeled normal DNA nucleotides, ^{32}P$_n$, and contaminants while the adducts are being retained at or close to OR. Subsequent separation of the adducts is conducted by development in D3 and D4 with 3 M lithium formate, 7–8.5 M urea (pH 3.5), and 0.8 M LiCl, 0.5 M Tris-HCl, 7–8.5 M urea (pH 8.0), respectively.

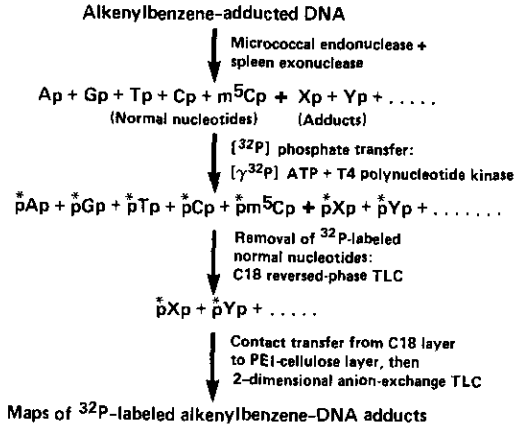

FIGURE 4. In the ^{32}P-postlabeling assay for alkenylbenzene-DNA adducts, reversed-phase (C18) TLC is substituted for anion-exchange TLC to separate normal DNA nucleotides from adducts.

bisphosphates (I). ^{32}P-labeling of nucleotides may be conducted under standard conditions (employing excess ATP over DNA-P) or under adduct intensification (ATP-deficient) conditions, as shown in Figure 2. The latter conditions afford the preferential labeling of many aromatic carcinogen–DNA adducts, which results in a greatly increased sensitivity of adduct detection (*7*). In addition to PEI–cellulose TLC, reversed-phase TLC on octadecylsilane (C18) layers was found to separate the normal nucleotides from the adduct nucleotides (*6*). To remove the labeled normal nucleotides and resolve the [^{32}P]adducts, a four-directional (4-D) anion-exchange PEI–cellulose TLC system was developed (Fig. 3). In this procedure, freshly prepared labeled digest (14–18 μCi/μL) is being applied slowly to the origin (Fig. 3, OR) located close to the center of the thin-layer sheet (20 × 20 cm). Development is begun immediately after sample application without drying of the origin area. Conditions for the various developments have been indicated in the legend of Figure 3. This procedure has been applied successfully to aromatic carcinogens having two to six aromatic rings; in the case of less aromatic carcinogens (such as alkenylbenzenes, sterigmatocystin, and aflatoxin B_1), removal of normal nucleotides from the adducts was best accomplished by C18 reversed-phase TLC (see Fig. 4).

The lower limit of adduct detection was found to be 1 adduct in (3.5–6) × 10^7 nucleotides for the standard 4-D PEI–cellulose TLC procedure, while one adduct in about 10^8 nucleotides could be detected if removal of normal nucleotides from the adduct nucleotides was carried out by reversed-phase TLC, the increase in sensitivity being due to reduced background radioactivity (*6*). The technique entails the simultaneous labeling of

normal and adduct nucleotides, thereby enabling the accurate quantitation of DNA adduct levels. It is also possible to isolate the adducts first and then label them in the virtual absence of normal nucleotides, a technique that affords an increase in sensitivity of detection to one adduct in about 10^{10} normal nucleotides (K. Randerath and E. Randerath, manuscript submitted).

Some examples of applications of the postlabeling method follow; additional examples have been described (*4–6*). The method was applied to skin DNA from mice treated topically with the polycyclic aromatic hydrocarbons benzo(a)pyrene (BP), 7,12-dimethylbenz(a)-anthracene (DMBA), and 3-methylcholanthrene (MC), respectively. As shown by autoradiography (Fig. 5), a large number of ^{32}P-labeled MC–DNA adducts was detected in digests of DNA obtained from mouse skin at several time points after carcinogen treatment. Four of these adducts (spots 4, 7, 9, and 10) were highly persistent (Figs. 5*c*, 5*d*). As shown in Figure 6, substantial removal of MC–DNA adducts occurred during the first 2 weeks after carcinogen application, while adducts remaining thereafter underwent little change. Analogous results were reported for BP–DNA and DMBA–DNA adducts (*5*), with some adducts persisting in mouse epidermis and dermis for about 1 year after a single topical carcinogen application (*7*). These results raise the possibility that the persistent adducts occupy specific genomic sites in quiescent cells where they may not be amenable to repair because of localized conformational alterations of DNA or shielding by associated proteins.

In Figure 7, application of the ^{32}P-postlabeling method to rat liver DNA after exposure *in vivo* to the mycotoxins aflatoxin B_1 and sterigmatocystin, respectively, is illustrated. These compounds are known potent hepatocarcinogens (*8,9*), and *in vivo* DNA binding of aflatoxin B_1 has been studied extensively (*10,11*), but no such data were available for sterigmatocystin–DNA binding *in vivo*. Our studies on these mycotoxins revealed that the adducts seen on ^{32}P-labeled fingerprints (Fig. 7) were mostly oligo- (di- and tri-)nucleotides, because the enzymatic digestion under standard conditions (*4*) of DNA containing such adducts led to the formation of oligonucleotides (mostly di- and trinucleotides) rather than to the formation of the usual mononucleotide adducts (*12*). Sterigmatocystin–DNA adducts were detectable in rat liver DNA as late as 3.5

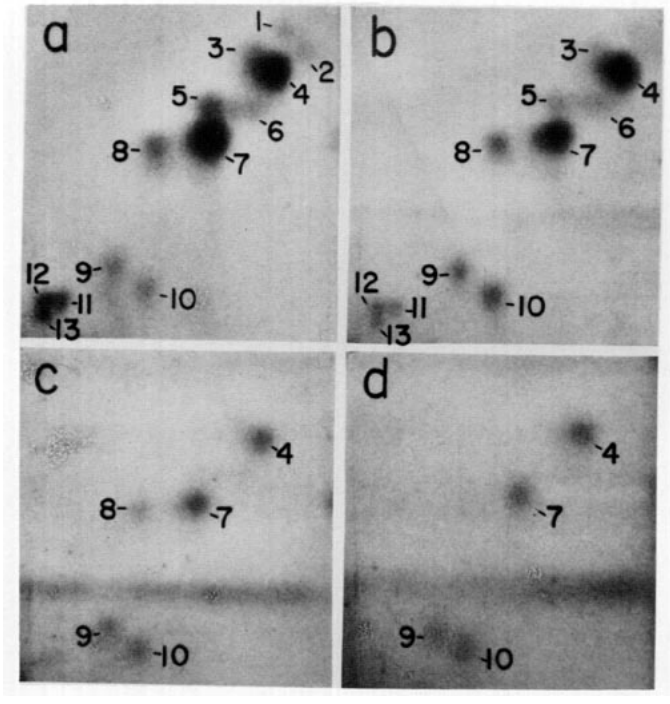

FIGURE 5. Maps of ^{32}P-labeled digests of mouse skin DNA isolated at (*a*) 1 day, (*b*) 6 days, (*c*) 14 days, and (*d*) 28 days after topical application of four 1.2-μmole doses of 3-methylcholanthrene in 200 μL acetone each. Digest of DNA from control mice that had received acetone only did not give any of the numbered spots. Separation was by the standard procedure (Fig. 3), and spots were visualized by autoradiography.

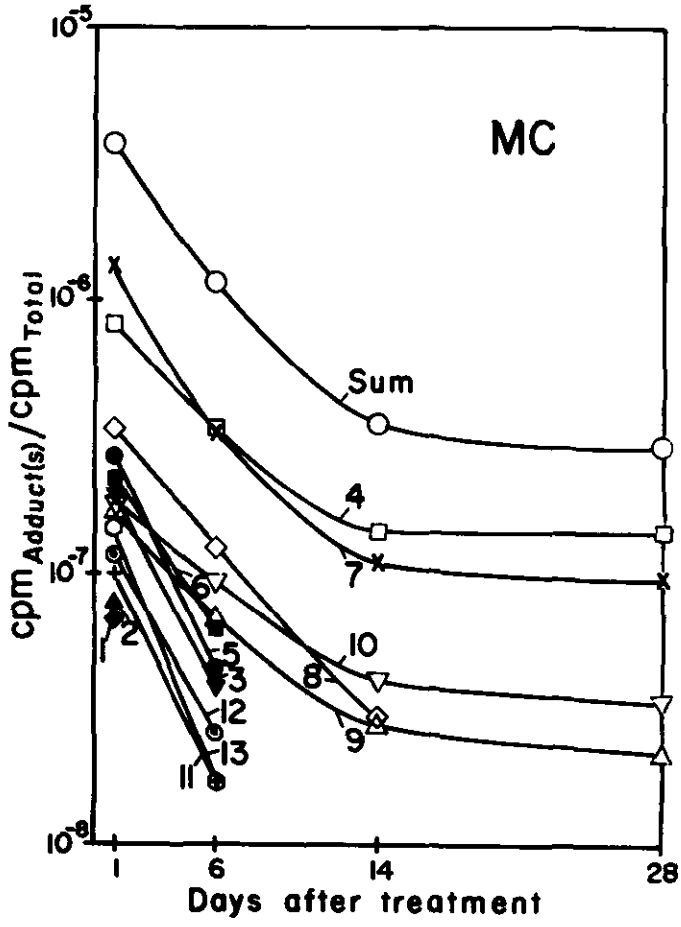

FIGURE 6. Time course of relative adduct labeling observed in the experiment illustrated in Fig. 5. A RAL value of 10^{-6} corresponds to a level of 3 fmole adduct/μg of DNA. Assays were performed by Cerenkov counting of excised spots. Relative standard deviations were $< \pm 15\%$.

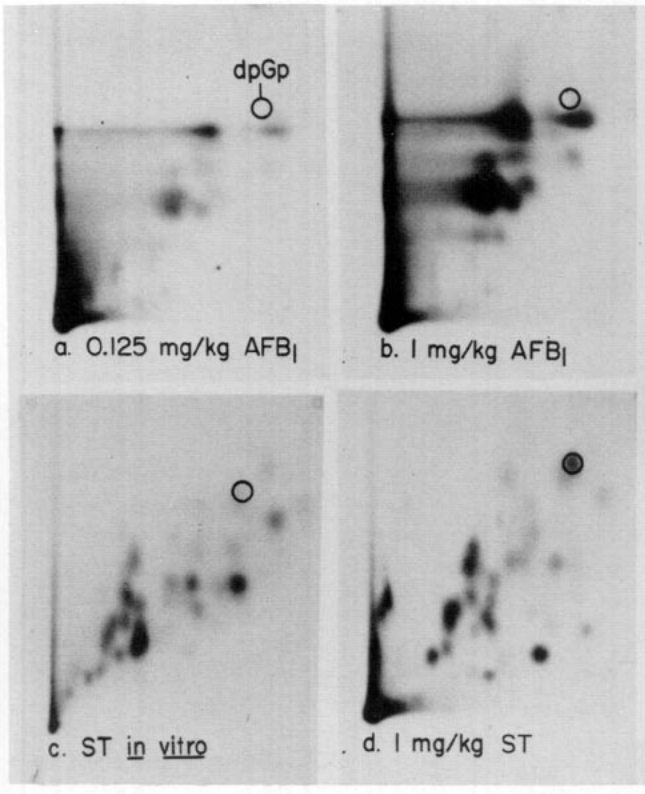

FIGURE 7. PEI–cellulose thin-layer maps of (*a,b*) aflatoxin B_1 (AFB_1)–DNA and (*c,d*) sterigmatocystin (ST)–DNA adducts; (*a, b, d*) obtained from DNA modified *in vivo*; (*c*) from DNA modified *in vitro* with sterigmatocystin in the presence of *m*-chloroperoxybenzoic acid under conditions similar to those used by Martin and Garner (*11*) for chemical modification of DNA with aflatoxin B_1. For the *in vivo* samples, rat liver DNA was isolated 2 hr after IP injection of the indicated dose of carcinogen, dissolved in 0.2 mL dimethyl sulfoxide. Adduct purification was by C18 reversed-phase TLC. Final mapping was by two-dimensional PEI–cellulose TLC in 3 M ammonium formate (pH 3.5) and 1.15 M Tris-HCl (pH 8.0).

months after injection of a single dose (9 mg/kg) of sterigmatocystin to male Fischer rats (*12*). When equal doses were compared, sterigmatocystin led to an approximately 10 times lower level of DNA modification than did aflatoxin B_1, in accord with a close to 10-fold lower carcinogenic potency of the former compound as compared to the latter (*9*).

We have applied the ^{32}P-postlabeling test to the analysis of the binding of a series of carcinogenic and noncarcinogenic alkenylbenzenes (*13*) to adult (*14*) and newborn (*15*) mouse liver DNA. Extensive carcinogenicity studies by Miller et al. (*13*) in mice had shown that, among these compounds, only three, i.e., safrole, estragole, and methyleugenol, were hepatocarcinogenic in the test animals. We wished to ascertain by ^{32}P-postlabeling assay whether a correlation existed between the DNA-binding activities of these compounds in mouse liver and their biological activity or lack of activity in assays for carcinogenicity in this organ. As illustrated in Figure 8, our results showed that the known hepatocarcinogens exhibited the strongest binding to mouse liver DNA 24 hr after IP administration of a 10-mg dose (one adduct in 10,000–15,000 DNA nucleotides or 200–300 fmole adduct/μg DNA), while the other structurally related alkenylbenzenes, except eugenol, all bound to mouse liver DNA also, but at lower levels.

In Table 1, most of the compounds we have studied thus far are listed, together with the tissues investigated, the number of adducts detected for each compound, and an estimation of total adduct level for each compound. With the exception of anthracene, pyrene, and perylene, all the compounds gave rise to ^{32}P-labeled adducts at the levels indicated. It appears noteworthy that within the group of polycyclic aromatic hydrocarbons, a good correlation was observed between the carcinogenic potency of individual compounds for mouse skin (*16,17*) and their binding levels to mouse skin DNA. In particular, we failed to detect DNA binding of the noncarcinogens anthracene, pyrene, and perylene (at a sensitivity of detection of one adduct in about 10^9 nucleotides), while the strong carcinogens BP, DMBA, and MC, exhibited the highest levels of DNA binding.

The illustrations provided in this article and elsewhere (*4–6*) demonstrate that each compound gives rise to a characteristic fingerprint of ^{32}P-labeled adduct derivatives on PEI–cellulose maps. Therefore, it is possible on the basis of such fingerprints, to identify the carcinogen to which the particular DNA had been exposed *in vivo*.

The adduct intensification version of the ^{32}P-postlabeling method was recently applied to detect adducts in human DNA from oral mucosa (B. Dunn, H. F. Stich, H. P. Agrawal, E. Randerath and K. Randerath, unpublished experiments) and human placenta (E. Randerath, R. Everson, R. Santella and K. Randerath, unpublished experiments) of smokers. This work showed that the method was applicable to the detection of adducts in DNA from humans and may thus become a tool for the detection of genetic damage in cells and tissues from humans exposed to carcinogenic/mutagenic chemicals.

Discussion

In this article a recently developed ^{32}P-postlabeling method for the analysis of carcinogen–nucleic acid adducts has been reviewed. While we have focused mainly on the analysis of carcinogen–DNA adducts, the method is also applicable to carcinogen–RNA adducts. The salient features of the new ^{32}P-postlabeling test for covalent DNA binding of chemicals can be summarized as follows.

The method enables the detection of minute amounts of adducts formed by the reaction of DNA with nonradioactive chemicals. At least in principle, therefore, DNA binding of any chemical can be assayed.

Since radioactive test chemicals are not required for *in vivo* studies, the assay is less expensive than assays employing radioactive chemicals.

It is applicable to individual compounds and to mixtures.

The chemical identity of test compounds and/or ad-

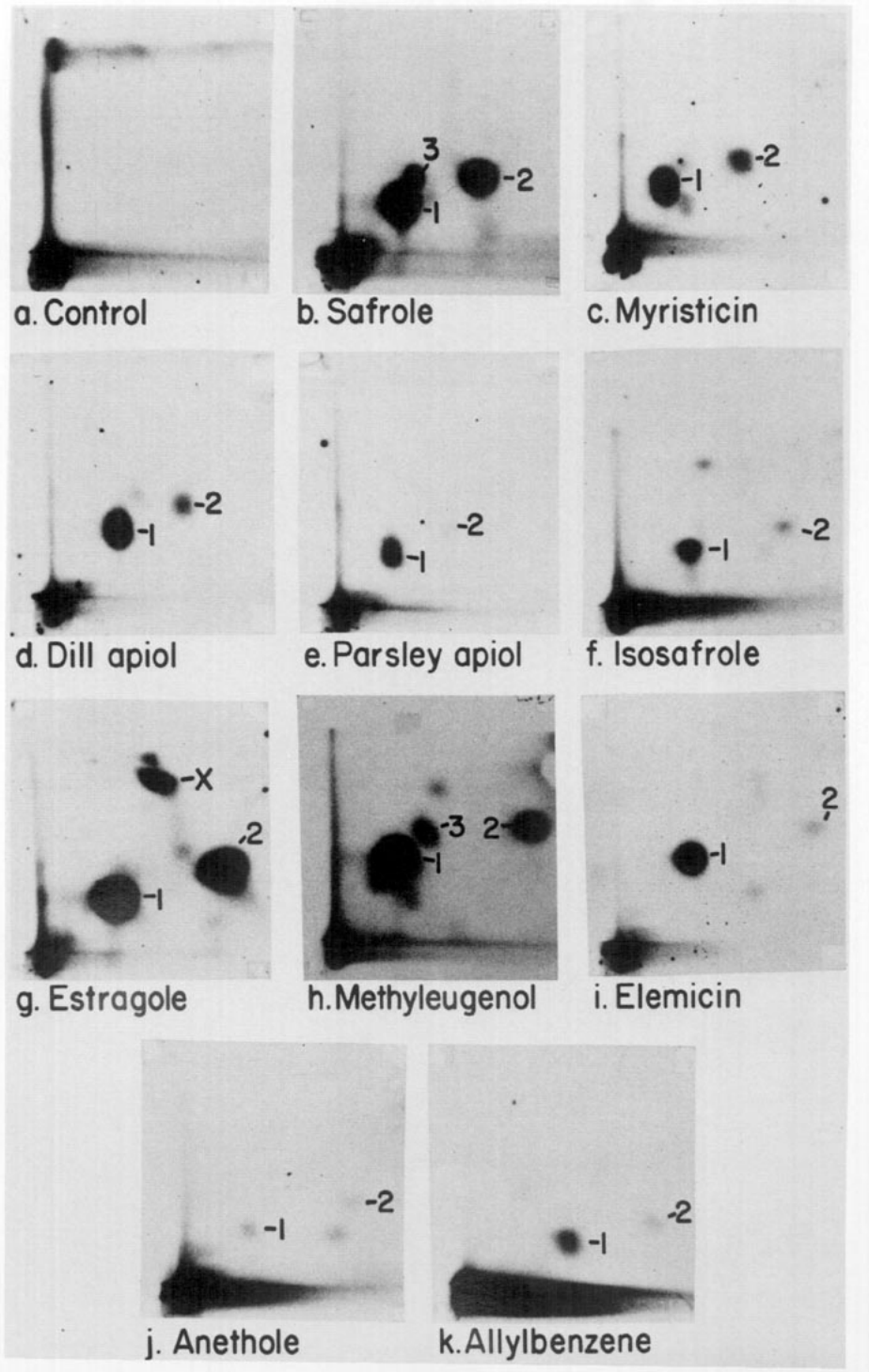

FIGURE 8. Maps of ^{32}P-labeled alkenylbenzene–DNA adducts from the livers of female CD-1 mice treated with a single 10-mg dose of the indicated test chemicals in tricaprylin for 24 hr. The adducts were purified by reversed-phase TLC (Fig. 4), resolved by PEI–cellulose anion-exchange TLC, and detected by autoradiography. Control (*a*), autoradiogram from liver DNA of mice treated with tricaprylin only. Spots of ^{32}P-labeled 3′,5′-bisphosphates of adducted deoxyribonucleosides are numbered 1, 2, and 3. X, unknown adduct. Autoradiography at −80° was performed for 12–15 hr, except for anethole (30 hr) and allylbenzene (20 hr) (*14*).

ducts does not have to be known for DNA binding to be detected.

If the fingerprint patterns obtained with pure chemicals or mixtures are known, then such fingerprints enable the identification of the binding chemical or mixture to which the DNA had been originally exposed.

Small (microgram) amounts of DNA are required for analysis.

The method is highly sensitive, in the case of aromatic adducts enabling the detection of a few adducts per

mammalian genome in a cell population or a tissue.

It can be used for accurate quantitation.

It is potentially useful for investigating the repair, removal, and loss of adducts from cell or tissue DNA; the effects of anticarcinogens, chemopreventive agents, and/or metabolic inhibitors on adduct formation and persistence can also be studied.

The method appears applicable to DNA from humans exposed to gene-altering chemicals and thus constitutes a tool for monitoring human exposure to such compounds.

The results reviewed in this article and by others (*18*) suggest that chemicals that are capable of forming covalent bonds with DNA in mammalian tissues are likely to be carcinogenic. In combination with chronic bioassays for carcinogenicity, the ^{32}P-postlabeling assay may thus be useful for answering the question as to whether covalent bond formation between a chemical and DNA *in vivo*, by itself, indicates carcinogenic potential of the particular chemical. More test chemicals need to be investigated to give a definite answer to this important question.

Included in our analyses were several compounds that, though being structurally related to known carcinogens, have not been found to date to be carcinogenic in chronic animal bioassays, i.e., 4-acetylaminofluorene, anthracene, pyrene, perylene, myristicin, dill apiol, parsley apiol, elemicin, anethole, and allylbenzene. With the exception of the polycyclic aromatic hydrocarbons, anthracene, pyrene, and perylene, DNA binding of these compounds *in vivo* was detected by ^{32}P-postlabeling assay, but was found to occur to a much lesser extent than that of the structurally related carcinogens. For example, the binding to rat liver DNA of 4-acetylaminofluorene was below that of 2-acetylaminofluorene by a factor of about 400 fold, and the noncarcinogenic alkenylbenzenes [myristicin, dill apiol, parsley apiol, elemicin, anethole, and allylbenzene (*13*)] bound to mouse liver DNA 4 to 200 times less than their carcinogenic counterparts (safrole, estragole, and methyleugenol) (*14*) These results demonstrate that the ^{32}P-postlabeling test is capable of detecting weakly genotoxic compounds, which either are not carcinogenic or whose carcinogenicity is not readily shown in animal bioassays. A compound such as myristicin, which binds only ca. 4 fold less than the structurally related hepatocarcinogen safrole to mouse liver DNA (*14*), but has not been shown to induce cancer in mouse bioassays (*13*), was found to exhibit substantial genotoxic activity in mouse liver *in vivo*. This raises the question as to whether the compound actually lacks any carcinogenic activity in spite of its DNA-damaging potential or whether bioassays conducted under different conditions would show its carcinogenicity.

Ashby (*19*) has recommended that a substantial proportion of the resources currently earmarked for chronic carcinogenicity bioassays might rather be employed in short-term *in vivo* evaluations in rodents of chemicals known to be genotoxic from various *in vitro* tests. On the basis of our results, the ^{32}P-postlabeling assay appears suitable to serve as such a short-term test for the detection of carcinogen-DNA adducts in animal tissues, i.e., as an assay for potential carcinogenicity of chemicals.

We have also pointed out in this article that the ^{32}P-postlabeling technique represents a tool to assay an important property of chemical carcinogens, i.e., the formation of persistent DNA adducts, which may play a crucial role in carcinogenesis. In using the test for the detection of covalent binding of chemicals to DNA, one should therefore also include an evaluation of adduct persistence. Compounds such as 7,12-dimethylbenz(a)anthracene or sterigmatocystin, which give rise in animals to highly persistent, essentially nonreparable adducts, induce irreversible toxic effects in the genetic material of mammals *in vivo*; it would appear prudent to preclude or minimize human exposure to such chemicals by appropriate regulatory measures. Since the ^{32}P-postlabeling assay makes possible the detection and quantitation of covalent binding of chemicals to DNA, as well as an analysis of the persistence of DNA lesions, it may become an important tool for risk assessment of genotoxic chemicals.

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REFERENCES

1. Lutz, W. K. *In vivo* covalent binding of organic chemicals to DNA as a quantitative indicator in the process of chemical carcinogenesis. Mutat. Res. 65: 289–356 (1979).
2. Lutz, W. K. The covalent binding index—DNA binding *in vivo* as a quantitative indicator for genotoxicity. In: Indicators of Genotoxic Exposure (Banbury Report 13), (B. A. Bridges, B. E. Butterworth and I. B. Weinstein, Eds.), Cold Spring Harbor Laboratory, 1982, pp. 189–202.
3. Randerath, K., Reddy, M. V., and Gupta, R. C. ^{32}P-labeling test for DNA damage. Proc. Natl. Acad. Sci. (U.S.) 78: 6126–6129 (1981).
4. Gupta, R. C., Reddy, M. V., and Randerath, K. ^{32}P-postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. Carcinogenesis 3: 1081–1092 (1982).
5. Randerath, E., Agrawal, H. P., Reddy, M. V., and Randerath, K. Highly persistent polycyclic aromatic hydrocarbon-DNA adducts in mouse skin: detection by ^{32}P-postlabeling analysis. Cancer Letters 19: 231–239 (1983).
6. Reddy, M. V., Gupta, R. C., Randerath, E., and Randerath, K. ^{32}P-postlabeling test for covalent DNA binding of chemicals *in vivo*: application to a variety of aromatic carcinogens and methylating agents. Carcinogenesis 5: 231–243 (1984).
7. Randerath, E, Agrawal, H. P., Weaver, J. A., Bordelon, C. B., and Randerath, K. ^{32}P-postlabeling analysis of DNA adducts persisting for up to 42 weeks in the skin, epidermis, and dermis of mice treated topically with 7,12-dimethylbenz(a)anthracene. Carcinogenesis, in press.
8. Wogan, G. N. Aflatoxin carcinogenesis. In: Methods in Cancer Res., Vol. 7 (H. Busch, Ed.), Academic Press, New York, 1973, pp. 309–344.
9. Purchase, I. F. H., and van der Watt, J. J. Carcinogenicity of sterigmatocystin. Food Cosmet. Toxicol. 8: 289–295 (1970).
10. Essigman, J. M., Croy, R. G., Bennett, R. A., and Wogan, G. N. Metabolic activation of aflatoxin B_1: patterns of DNA adduct formation, removal, and excretion in relation to carcinogenesis. Drug Metabol. Rev. 13: 581–602 (1982).

11. Martin, C. N., and Garner, R. C. Aflatoxin B-oxide generated by chemical or enzymic oxidation of aflatoxin B_1 causes guanine substitution in nucleic acids. Nature 267: 863–865 (1977).
12. Reddy, M. V., Irvin, T. R., and Randerath, K. Formation and persistance of sterigmatocystin—DNA adducts in rat liver determined via ^{32}P-postlabeling analysis. Mutat. Res., in press.
13. Miller, E. C., Swanson, A. B., Phillips, D. H., Fletcher, T. L., Liem, A., and Miller, J. A. Structure-activity studies of the carcinogenicities in the mouse and rat of some naturally occurring and synthetic alkenylbenzene derivatives related to safrole and estragole. Cancer Res. 43: 1124–1134 (1983).
14. Randerath, K., Haglund, R. E., Phillips, D. H., and Reddy, M. V. ^{32}P-postlabeling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole, and other naturally-occurring alkenylbenzenes. I. Adult female CD-1 mice. Carcinogenesis 5: 1613–1622 (1984).
15. Phillips, D. H., Reddy, M. V., and Randerath, K. ^{32}P-postlabeling analysis of DNA adducts formed in the livers of animals treated with safrole, estragole, and other naturally-occurring alkenylbenzenes. II. Newborn male $B6C3F_1$ mice. Carcinogenesis 5: 1623–1628 (1984).
16. Dipple, A. Polynuclear aromatic carcinogens. In: Chemical Carcinogens (C. E. Searle, Ed.), American Chemical Society, Washington, DC, 1976, pp. 245–314.
17. Phillips, D. H., Grover, P. L., and Sims, P. A quantitative determination of the covalent binding of a series of polycyclic hydrocarbons to DNA in mouse skin. Int. J. Cancer. 23: 201–208 (1979).
18. Hemminki, K. Nucleic acid adducts of chemical carcinogens and mutagens. Arch. Toxicol. 52: 249–285 (1983).
19. Ashby, J. The unique role of rodents in the detection of possible human carcinogens and mutagens. Mutat. Res. 115: 177–213 (1983).